Changes in the soluble nitrogen fraction of milk throughout PDO Grana Padano cheese-making

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ABSTRACT

The behavior of soluble nitrogen compounds during Grana Padano cheese-making was studied at eight dairies. Raw milk, skimmed milk, sweet whey and the derived natural whey culture, collected from 24 processes, were analyzed for soluble whey proteins (α-La and β-Lg), proteose-peptones (PP), small peptides (SP), caseinomacropeptides (CMPs), and free amino acids (FAA). The PP fraction increased during milk natural creaming, then part of it was selectively retained in the curd and the rest degraded in the first few hours of whey fermentation, together with α-La, CMPs and part of SP. Features outlined for the whey culture have been confirmed on 30 samples collected at six different dairies. A time course study of the whey fermentation showed that degradation of α-La begins when the pH drops below 4, whereas β-Lg content did not change. Uptake of specific FAA is shown to support the initial growth of lactic acid bacteria in whey.

1. Introduction

Grana Padano PDO cheese is manufactured in a defined geographical area of northern Italy using the traditional process described in the product specification (European Parliament and Council, 2012). Holstein-Friesian cows make up almost 95% of the dairy herds providing milk for Grana Padano manufacture. Corn silage represents the most important part of the cows' diet all year round, whereas some differences may exist in concentrate supplementation, depending upon the season (Borreani et al., 2013).

Raw milk is regularly collected at farms within 12 h after milking and, notwithstanding hygiene prescriptions (European Commission, 2004), must not be cooled below 8°C since refrigeration decreases both natural creaming and rennet coagulation suitability, and alters the native microbial balance (Caplan et al., 2013; Montel et al., 2014; Raats et al., 2011). At the dairy, milk is partly skimmed by natural creaming, then transferred to a traditional copper vat; natural whey starter is added and coagulation occurs at 32-33°C. The curd is cut into very small granules, cooked to 53-56°C and left to compact at the bottom of the vat for 40-60 min before extraction and molding. Part of the whey (at pH 6.2-6.4) is taken from the vat and incubated to obtain the natural whey starter for cheese-making the next day. The whey starter for Grana Padano (titratable acidity 28-30 °SH per 50 mL, pH 3.3-3.6) mainly contains thermophilic strains of lactic acid bacteria (LAB), i.e. *Lactobacillus helveticus* (60-80%), *Lactobacillus delbrueckii* ssp*. lactis* plus *Lactobacillus delbrueckii* ssp*. bulgaricus* (10-40%), and *Streptococcus thermophilus* (1-20%), with a total count in the order of 8-9 log cfu mL^{-1} (Rossetti et al., 2008; Santarelli et al., 2008; Cremonesi et al., 2011). Other wild strains, including some heterofermentative species such as *Lactobacillus fermentum, Lactobacillus rhamnosus, Lactobacillus casei, Pediococcus acidilactici,* represent minor species, commonly considered as nonstarter LAB (NSLAB) (Gatti et al., 2014). The cheese curd is kept in a mold for 48

47 h to allow cooling and acidification by LAB fermentation before 18-20 d of salting in brine. All of
48 these steps are very well characterized in their microbiological (Giraffa et al., 1998; Gatti et al.,
49 2006) and technological features (Pellegrino et al., 1997).

50 Several analytical parameters currently adopted in identity assessment and quality control of
51 Grana Padano cheese on the retail market are based on these studies (Cattaneo et al., 2008;
52 Masotti et al., 2010). In contrast, much less attention has been paid to clarifying how the complex
53 microbiota of raw milk affects milk components throughout the cheese-making process. In
54 particular, very few studies have been dedicated to investigating how milk components change
55 during natural creaming and in-vat working, or how whey components change during
56 fermentation (Bosi et al., 1990). Considering the nutritional requirements of LAB, besides lactose,
57 the soluble nitrogen compounds (SNCs) represent the most important source of energy for
58 growth.

59 This paper investigates how the soluble nitrogen fraction of raw milk is modified throughout milk
60 pre-treatment and in-vat working and during the subsequent fermentation of the cheese whey in
61 the manufacture of Grana Padano. This knowledge is interesting for cheese in general but acquires
62 particular relevance in the case of hard cheeses, whose manufacturing process (from milk arrival
63 at the dairy to curd molding) may last up to 20-24 h. For this purpose, samples were collected
64 along the cheese-making process at different Grana Padano dairies, and the SNCs, namely
65 individual whey proteins, peptones, peptides and free amino acids, were evaluated. Seasonal
66 variability was considered as well.

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2. Materials and methods

2.1 Milk, whey and whey culture samples

A total of 24 cheese manufacturing processes were studied at eight dairies of the Consorzio di Tutela del Formaggio Grana Padano DOP in different periods of the year (i.e. late spring-summer and late autumn-winter). The same sampling procedure was strictly followed. Whole raw milk (WM) was taken from the collection tank (bulk >20 tons) on arrival at the dairy, and the corresponding skimmed (fat 2.3-2.4%) milk (SM) obtained by natural creaming was collected for coagulation. At the end of in-vat working, sweet whey (SW) was taken from the same vat after curd extraction and the derived natural whey culture (WC) was sampled after 20-22 h of fermentation. In addition, 30 samples of WC were collected on different days from six other dairies of the Grana Padano production area and analyzed. Finally, at one of the involved dairies, samples were taken from the whey fermentation tank at 0, 1, 2, 3, 4, 6, 9, 12, 20 h during the regular incubation process on two different days, while temperature and pH were recorded. All of the samples were immediately frozen and kept at -20°C until analysis. Standard plate count (SPC) and somatic cell count (SCC) data were kindly provided by the dairies.

2.2 Chemicals

Pure α-lactalbumin (α-La) (code L6010) and β-lactoglobulin (β-Lg) (code L0130) and single amino acids were purchased from Sigma-Aldrich (Milan, Italy). Nynhydrin was purchased from Biochrom Ltd (Cambridge, UK). All chemicals were of analytical grade.

2.3 Analytical methods

Contents of soluble whey proteins, proteose-peptones (PP), caseinomacropeptides (CMPs) and small peptides (SP) were determined by HPLC in WM, SM, SW and WC samples using the same preparation procedure. The samples were adjusted to pH 4.6 with either 2N HCl or 2N NaOH,

centrifuged (2000 *g* for 15 min) and the supernatant was filtered through a 0.22 µm membrane filter (Millipore, Vimodrone, Italy) before HPLC analysis. The HPLC system consisted of an Alliance module equipped with a 996 DAD detector (Waters, Milford, MA, USA) operated at 205 nm and the data were recorded and integrated using Empower^{TM} software (Waters). The chromatographic column PLRP-S (250 x 4.6 mm, 300 Å pore size, 5 µm particle size) (Varian Medical System, Milan, Italy) was kept at 40°C. Chromatographic conditions described by the ISO Standard 13875 (2005) were adopted. The elution gradient (De Noni et al., 2007) allowed the separation of SP (eluting from 4 to 7 min) and PP (from 11 to 15 min) (Fig. 1). The two peaks corresponding to the non-glycosylated CMPs A and B were identified according to Thoma et al. (2006). Soluble α-La and β-Lg were quantified by an external standard method using commercially pure proteins to obtain calibration curves in the range 100-3000 mg L^{-1} (r^2>0.98 for both the proteins). Quantification of PP, CMPs and SP was achieved using the calibration curve of α-La. Free amino acids (FAA) were determined on the same filtrates after 1:1 dilution with lithium citrate buffer at pH 2.2 and further filtration through 0.2 µm filter (Millipore). The chromatographic separation was carried out on a Biochrom 30+ (Biochrom Ltd, Cambridge, UK) automatic amino acid analyzer operated under the conditions provided by the manufacturer. These employ an eight-step elution program with lithium citrate buffers of increasing pH and ionic strength, post-column derivatization with ninhydrin, and detection at 440 and 570 nm. The quantification was carried out using four-level calibration lines of the 21 amino acids in the range 0.75-22.5 mg L^{-1} and using norleucine (Sigma-Aldrich) as an internal standard. Repeatability values of ISO Standard 13903 (2005) were fulfilled.

2.4 Statistical analyses

All analyses were carried out in duplicate and mean values and standard deviations were considered. Ranges and coefficients of variation (CV) were reported to express overall variability of

115 the different types of samples for the tested parameters. Comparisons were made by Tukey's test
116 and ANOVA, and $P<0.01$ was considered significant. Statistical analyses were carried out using
117 Minitab software (release 14, 2004; State College, PA, USA).

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119 **3. Results and discussion**

120 A systematic study was firstly carried out on 24 cheese manufacturing processes to evaluate how
121 the SNCs change through technological, enzymatic and microbiological factors. To reach this goal,
122 we used specific analytical techniques to quantify the main constituents of SNCs with high
123 accuracy. As we have shown in previous work (De Noni et al., 2007; Cattaneo et al. 2014), SP (MW
124 <10 KDa), PP, as well as the main native whey proteins, can all be evaluated by HPLC in a single run
125 (Fig. 1). The presence of CMPs could also be detected (Thoma et al., 2006) and quantified in whey
126 samples. In addition, the pattern of FAA was systematically studied for the first time in samples of
127 cheese milk and in the derived SW and WC. The analysis was carried out by ion exchange
128 chromatography, since this technique proved to be more reliable than HPLC for this purpose (ISO,
129 2005). Data obtained for individual SNCs are summarized in Fig. 2.

130 *3.1 Variability of soluble nitrogen composition of raw milk intended for Grana Padano cheese-*
131 *making*

132 Amongst the whey proteins, only α-La and β-Lg were considered here, since these are less
133 influenced by stage of lactation and health conditions of the cows. The coefficient of variation (CV)
134 was 4% and 9% for the content of α-La and β-Lg, respectively (Fig. 2), independent of the season
135 (Table 1). The levels are in accordance with those reported by other authors (Schlimme et al.,
136 1996; Wedholm et al., 2006, Stergiadis et al., 2013). As expected, a much higher variability was

137 found for PP and SP, the contents of which are dependent upon enzyme activities. Although all
138 samples were collected within 12 h after milking, PP levels ranged between 280 and 655 mg L^{-1} (CV
139 22%) (Fig. 2). The content of PP in raw milk increases during storage as a result of the activity of
140 plasmin on β-casein. Furthermore, the rate of increase of PP content is positively correlated with
141 milk SCC because the complex activation system of plasminogen to plasmin is partially bound to
142 these cells (Ismail and Nielsen, 2010). These features explain the very wide range of PP levels (500-
143 3000 mg L^{-1}) in raw milk reported in the literature (Pâquet 1989; Van Boekel & Crijns, 1994; Merin
144 et al. 2008). Data in the current study falls at the lowest end of this range, suggesting that
145 management of milk collection in the Grana Padano system does not promote extensive plasmin
146 activity which is detrimental to rennet coagulation. Levels of SP were in the range from 255 to 722
147 mg L^{-1}, with CV=28% (Fig. 2), comparable with our previous data. Noticeably, data in Table 1 show
148 that, since raw milk is only partly cooled before collection, high temperatures in summer strongly
149 affect variability (CV) of SP content. In contrast, PP contents shift to slightly higher levels, due to
150 the physiologically higher values of SCC (Bertocchi et al., 2014), whereas variability does not
151 change.

152 Total FAA levels were in the range 63-90 mg L^{-1}, with CV=12% (Fig. 2) and, contrary to what has
153 been observed for SP, variability was independent from the season (Table 1). These levels are
154 comparable to those reported by Csapò et al. (1995) on Holstein cows' milk and by Mills and
155 Thomas (1981) on cows' milk, i.e. 34 and 69 mg L^{-1} respectively. The pattern of FAA is shown in
156 Table 2. Remarkably, glutamic acid represents 30% of the FAA on average, whereas some other
157 amino acids (glycine, alanine, aspartic acid, arginine, glutamine, valine, lysine, proline) are present
158 at lower levels, comparable with each other. Surprisingly, we also detected small amounts (<1 mg
159 L^{-1}) of FAA, namely ornithine, citrulline and y-amino-butyric acid, which are not present in milk
160 proteins and might originate from early microbial metabolism.

161 *3.2 Proteolysis during milk preparation and in vat processing*

162 An increase (P<0.01) of PP content of 20% was observed for all of the semi-skimmed milk samples,
163 with respect to the corresponding parent whole milk (Fig. 2), as a result of plasmin activity taking
164 place during gravity separation of fat. At the temperatures used (usually 10-16°C), enzyme
165 activities are not fully inhibited. This limited proteolysis, in combination with slight acidification, is
166 considered to improve casein susceptibility to rennet coagulation (Resmini et al., 1982). In
167 contrast, extensive plasmin activity in cheese milk caused an increased nitrogen loss in whey,
168 although the nature of that fraction was not investigated (Mara et al., 1998). Hence, the pattern of
169 SNCs in cheese milk can provide an explanation of unexpectedly anomalous behavior upon
170 coagulation. Due to the bacteriological purification achieved (Dellaglio et al., 1969; Caplan et al.,
171 2013), no further protein or peptide degradation occurred in milk and hence both SP and FAA
172 levels remained almost unchanged (Fig. 2).

173 Considering that CMPs represent about 3% of casein weight (Thoma et al., 2006) and that about
174 20% of CMPs are not released in Grana Padano coagulation (Resmini et al., 1982), the levels of
175 CMPs we found in whey samples (Fig. 2) are consistent with what might be expected, although
176 they were calculated using the same response factor at 205 nm as for α-La. Due to the sharp
177 increase in temperature during vat working and the parallel dehydration of casein micelle surface
178 (O'Mahony & Fox, 2013), enzymes likely only facilitate specific (primary) protein hydrolysis. It has
179 been demonstrated (Sheehan et al., 2007; Masotti et al., 2010) that plasmin is not inactivated
180 during in-vat processing, and is able to hydrolyze β-casein, even during ripening of cheeses cooked
181 at high temperature, such as Grana Padano. However, the enzymatic activity did not occur during
182 curd cooking, probably due to the progressive aggregation of casein micelles through strong
183 hydrophobic interactions which may limit enzyme accessibility. The pattern we obtained for fresh

184 Grana Padano curd by capillary zone electrophoresis (CZE) (Cattaneo et al., 2008) fully overlaps
185 that of the vat milk for all the individual casein fractions, but not for κ-casein (not shown).
186 Unexpectedly, the level of PP in whey was much lower (P<0.01) than in the parent vat milk,
187 whereas the level of SP was about twice as high (Fig. 2). The increase of SP derives from both the
188 added WC and proteolytic activity of the most thermophilic LAB. Partial hydrolysis of PP cannot be
189 excluded to explain the lower levels we found in the SW. Van Boekel and Crijns (1994) showed
190 that, under laboratory conditions, the content of PP5 (the most hydrophobic among PP
191 components) in rennet whey changes depending upon pH of milk at coagulation, and a possible
192 association of this component with paracasein was hypothesized. Merin et al. (2008) report that
193 addition of PP components to milk increased the clotting time and curd firmness. Nevertheless, no
194 direct evidence is available in the literature for the selective retention of PP in cheese curd.
195 Preliminary results we obtained by CZE of Grana Padano cheese curd (not shown) point in this
196 direction. However, further investigation is needed to clarify this aspect, which is of high practical
197 interest as it directly affects cheese yield.

198 The content of FAA increased by 15-20% on average (P<0.05), confirming that primary proteolysis
199 mostly takes place in this step. Thermal conditions occurring during in-vat processing did not cause
200 denaturation of α-La and β-Lg (Fig. 2). From the HPLC chromatograms (Fig. 1), it can be seen that
201 more heat-sensitive whey proteins, i.e. bovine serum albumin and immunoglobulins, not
202 quantified in this study, did not undergo detectable denaturation. Besides whey proteins, the
203 content of soluble nitrogen molecules in the SW from Grana Padano cheese-making accounted for
204 approximately 2000 mg L^{-1} on average, i.e. two times the amount in vat milk. One fourth of this
205 amount is represented by CMPs. Obviously, the same SNCs present in the whey phase are also
206 retained in the extracted curd and will represent the initial nitrogen sources for LAB to grow
207 during the molding time.

208 *3.3 Proteolysis during the whey fermentation*

209 The composition of SNCs changed dramatically during the whey fermentation process (Fig. 2).
210 Lacking casein, SNCs represent a source of essential amino acids for LAB growth in whey. For the
211 first time, the behavior of the individual components has been evaluated in this study along the
212 same production process, from raw milk to the derived WC. This approach has allowed even minor
213 changes to be highlighted and quantitatively evaluated. The most relevant finding was that α-La
214 was almost completely hydrolyzed whereas β-Lg remained intact. This aspect is currently under
215 study, since published literature on the capability of LAB to degrade soluble whey proteins is
216 contradictory. Differences in tested strains and growing conditions can partially explain
217 discrepancies among studies (Bosi et al., 1990). Furthermore, to our knowledge no data are
218 available on soluble whey protein pattern in natural WC for Grana Padano cheese-making and in
219 natural starter cultures in general.

220 All non-protein SNCs were intensively degraded (P<0.01). Residual traces of CMPs and PP were
221 detected in WC, whereas the content of SP decreased by 40% on average but the range of values
222 found was very wide. This pool of low-MW peptides originates from the proteolytic activity of LAB
223 and hence individual peptides are continuously formed and hydrolyzed over time. Therefore, a
224 defined pattern of these components can not be established and can not give reliable information
225 on microbial growth behavior. In contrast, FAA represent more stable molecules, limited in
226 number and directly related to LAB metabolic pathways. As a result of LAB growth, the FAA
227 content increased by a factor 4 to 5 (Fig. 2). Recently, genomic studies are increasingly being used
228 to clarify the proteolytic systems of LAB and to identify specific proteinase and peptidase activities
229 relevant to cheese ripening (Liu et al., 2010; Broadbent et al., 2011). These studies will shed more

230 light on LAB growth in whey as well. However, direct evidence of the enzymes actually involved
231 can only be achieved by evaluating the FAA pattern modification.

232 The overall SNCs pattern we found in WC was fully confirmed on a larger number of samples
233 collected at six other Grana Padano cheese factories (Fig. 3), covering a wider area of the
234 production zone. The ranges of variation were the same as in Fig. 2 for all fractions, for the first
235 time showing that the SNC composition in natural WC of Grana Padano is well-defined, despite the
236 unavoidable variability in management conditions of the preparation process.

237 Finally, a time course study of the SW fermentation step was carried out. Two batches were
238 considered from the same factory to include the day-to-day variability. As shown in Fig. 4, PP and
239 SP were promptly hydrolyzed by LAB at the beginning of growth, since both represent more ready
240 nitrogen sources with respect to α-La and CMPs. Although the PP level dropped within the first
241 two hours, reaching a plateau at around 100 mg L^{-1}, SP level decreased more progressively
242 throughout the whole fermentation process. This behavior is likely the result of the progressive
243 hydrolysis of peptides into new ones, depending upon microbial protease and peptidase patterns,
244 hence confirms that the SP fraction is not reliable in characterizing the WC. It must be mentioned
245 that oligopeptide uptake systems have been identified in several LAB having different specificities
246 (Mills & Thomas, 1981; Slattery et al., 2010). However, these aspects have mainly been
247 investigated in milk or cheese, whereas the specific growth conditions considered here, i.e. in a
248 medium lacking in casein and obtained from a vat process which is highly stressing for these
249 organisms, need dedicated studies. Hydrolysis of high MW components, namely α-La and CMPs,
250 only began after 3-4 h of fermentation when the pH of the environment decreased below 4 (Fig.
251 4a). The drop in pH undoubtedly plays a role in enabling proteases to attack α-La, since it modifies
252 the molecule conformation (O'Mahony & Fox, 2013). The content of FAA showed an irregular

253 behavior in the first 5-6 h, afterwards increasing linearly, and was approximately five times higher
254 at the end of the incubation period. This increase may be partly attributed to cell lysis, although no
255 clear evidence of this phenomenon during whey fermentation is available from the literature,
256 since studies were mainly carried out in cheese or culture broth. Moreover, the mechanisms of
257 biosynthesis and metabolism of amino acids in LAB are still unclear. We have analyzed single
258 amino acids throughout whey fermentation and found that many of them behaved rather
259 differently (Fig. 5). Amino acids that are essential to LAB (e.g. leucine, serine, threonine, tyrosine,
260 methionine, arginine, glutamic acid), depending upon the species (Slattery et al., 2010; Broadbent
261 et al., 2011; Liu et al., 2010), are promptly taken up from the FAA pool to support the initial
262 growth phase. Some FAA are almost completely consumed within the first 2-3 hours, then start to
263 accumulate (Figs. 5-a and -b) as a result of different metabolic requirements of various LAB species
264 involved. After the initial uptake, free glutamic acid is released by the cells, but as soon as pH
265 drops, it is decarboxylated to γ-amino butyric acid (Fig. 5-c). Arginine uptake looks to be pH-
266 dependent as well (Fig. 5-d). After the initial uptake it accumulates in whey. However, as the
267 increase of acidity stresses the LAB, uptake starts again since arginine can be converted into
268 citrulline by intracellular enzymatic pathways to increase acid resistance. Apparently citrulline is
269 not released into the medium. Although confirmation with a larger number of samples is needed,
270 these observations demonstrate the importance of FAA, even in a peptide-rich environment. This
271 approach may contribute to understanding the complex amino acid biosynthetic and metabolic
272 pathways of LAB.

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Conclusions

Individual SNCs have been evaluated for the first time throughout the whole manufacturing process of Grana Padano cheese by using specific analytical methods. Natural creaming of raw milk and in-vat working proved to be relevant steps of the process contributing substrates for LAB to growth during subsequent whey fermentation. In particular, PP content increased by 25% during the first step whereas SP almost doubled in the second step. Amongst whey proteins, β-Lg remained stable throughout the whole manufacturing process, whereas α-La was completely hydrolyzed during whey fermentation. The evolution of the FAA pattern gave direct evidence for specific LAB activities and nutritional requirements. These results represent a useful complement to existing microbiological data for understanding the complex phenomena occurring during manufacturing of hard cheeses. Furthermore, our data may help to interpret the selective growth of various LAB species in a changing substrate (milk, sweet whey, acid whey) leading to different microbial balances throughout the process.

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297 **References**

298 Bertocchi, L., Vitali, A., Lacetera, N., Nardone, A., Varisco, G., & Bernabucci, U. (2014). Seasonal
299 variations in the composition of Holstein cow's milk and temperature-humidity index
300 relationship. *Animal, 8*, 667-674.

301 Borreani, G., Coppa, M., Revello-Chion, A., Comino, L., Giaccone, D., Ferlay, A., & Tabacco E.
302 (2013). Effect of different feeding strategies in intensive dairy farming systems on milk fatty
303 acid profiles, and implications on feeding costs in Italy. *Journal of Dairy Science, 96*, 6840-
304 6855.

305 Bosi, F., Bottazzi, V., Vescovo, M., Scolari, G.L., Battistotti, B. & Dellaglio, F. (1990). Lactic acid
306 bacteria for Grana cheese production. Part I. Technological characterization of thermophilic
307 rod lactic acid bacteria. *Scienza e Tecnica Lattiero-casearia, 41,* 105-136.

308 Broadbent, J.R., Cai, H., Larsen, R.L., Hughes, J.D., Welker, D.L., De Carvalho, V.G., Tompkins, T.A.,
309 Ardo, Y., Vogensen, F., De Lorentiis, A., Gatti, M., Neviani, E., & Steele, J.L. (2011). Genetic
310 diversity in proteolytic enzymes and amino acid metabolism among *Lactobacillus helveticus*
311 strains. *Journal of Dairy Science, 94,* 4313-4328.

312 Caplan, Z., Melilli, C., & Barbano, D.M. (2013). Gravity separation of fat, somatic cells, and bacteria
313 in raw and pasteurized milks. *Journal of Dairy Science*, *96*, 2011-2019.

314 Cattaneo, S., Hogenboom, J.A., Masotti, F., Rosi, V., Pellegrino, L., Resmini, P. (2008). Grated Grana
315 Padano cheese: new hints on how to control quality and recognize imitations. *Dairy Science*
316 *& Technology, 88*, 595-605.

317 Cattaneo, S., Stuknyté M., Pellegrino L., De Noni I. (2014). Targeted peptides for the quantitative
318 evaluation of casein plasminolysis in drinking milk. *Food Chemistry 155,* 179-185.

319 Csapò, J., Csapò-Kiss, Z., Stefler, J., Martin, T.G., & Nemethy S. (1995). Influence of mastitis on D-
320 amino acid content of milk. *Journal of Dairy Science, 78*, 2375-2381.

321 Cremonesi, P., Vanoni, L., Morandi, S., Silvetti, T., Castiglioni, B., & Brasca, M. (2011). Development
322 of a pentaplex PCR assay for the simultaneous detection of Streptococcus thermophilus,
323 Lactobacillus delbrueckii ssp. bulgaricus, L. delbrueckii ssp. lactis, L. helveticus, L. fermentum
324 in whey starter for Grana Padano cheese. *International Journal of Food Microbiology, 146*,
325 207-211.
326 Dellaglio, F., Stadhouders, J., & Hup, G. 1969. Distribution of bacteria between the bottom, middle,
327 and cream layers of creamed raw milk. *Netherland Milk & Dairy Journal, 23,* 140–145.
328 De Noni, I., Pellegrino, L., Cattaneo, S., & Resmini, P. (2007). HPLC of proteose peptones for
329 evaluating ageing of packaged pasteurized milk. *International Dairy Journal,17,* 12-19.
330 European Commission, 2004. Regulation No 853/2004 laying down the specific hygiene rules for
331 foods of animal origin. Official Journal L226, 26.6.2004, p 22.
332 European Parliament and Council, 2012. Regulation No 1151/2012 on quality schemes for
333 agricultural products and foodstuffs. Official Journal L343, 14.12.2012, p.1.
334 Gatti, M., Bottari, B., Lazzi, C., Neviani, E., & Mucchetti, G. (2014). Invited review: Microbial
335 evolution in raw-milk, long-ripened cheeses produced using natural whey starters. *Journal of*
336 *Dairy Science, 97*, 1-19.
337 Gatti, M., Bernini, V., Lazzi, C., & Neviani, E. (2006) Fluorescence microscopy for studying the
338 viability of microorganisms in natural whey starters. *Letters of Applied Microbiology, 42,* 338-
339 343.
340 Giraffa, G., Rossetti, G., Mucchetti, G., Addeo, F., & Neviani E. (1998). Influence of the temperature
341 gradient on the growth of thermophilic lactobacilli used as natural starters in Grana cheese.
342 *Journal of Dairy Science, 81*, 31-36.

343 ISO Standard 13875:2005 (IDF 178: 2005) Liquid milk - Determination of acid-soluble beta-
344 Lactoglobulin content - Reverse-phase HPLC method. Geneva, Switzerland: International
345 Organization for Standardization.
346 ISO Standard 13903:2005 Animal feeding stuffs - Determination of amino acids content. Geneva,
347 Switzerland: International Organization for Standardization.
348 Ismail, B., & Nielsen, S. S. (2010). Plasmin protease in milk: Current knowledge and relevance to
349 dairy industry. *Journal of Dairy Science, 93*, 4999–5009.
350 Liu, M., Bayjanov, J.R., Renckens, B., Nauta, A., & Siezen, R.J. (2010). The proteolytic system of
351 lactic acid bacteria revisited: a genomic comparison. *BMC Genomics*, 11, 36-51.
352 Mara, O., Roupie, C., Duffy, A., & Kelly, A.L. (1998).The curd-forming properties of milk as affected
353 by the action of plasmin. *International Dairy Journal, 8,* 807-812.
354 Masotti, F., Hogenboom, J.A., Rosi, V., De Noni, I., & Pellegrino, L. (2010). Proteolysis indices
355 related to cheese ripening and typicalness in PDO Grana Padano cheese. *International Dairy*
356 *Journal, 20*, 352-359.
357 Merin, U., Fleminger, G., Komanowski, J., Silanikove, N., Bernstein, S., & Leitner, G. (2008).
358 Subclinical udder infection with *Streptococcus dysgalactiae* impairs milk coagulation
359 properties: The emerging role of proteose peptones. *Dairy Science & Technology, 88,* 407-
360 419.
361 Mills, O.E. & Thomas, T.D. (1981). Nitrogen sources for growth of lactic Streptococci in milk. *New*
362 *Zealand Journal of Dairy Science and Technology, 16*, 43-55.
363 Montel, M.C., Buchin, S., Mallet, A., Delbes-Paus, C., Vuitton, D.A., Desmasures, N., & Berthier, F.
364 (2014). Traditional cheeses: Rich and diverse microbiota with associated benefits.
365 *International Journal of Food Microbiology, 177*, 136-154.

366 O'Mahony, J.A., & Fox, P.F. (2013). Milk proteins: introduction and historical aspects. In P.L.H.
367 McSweeney & P.F. Fox (Eds.). *Advanced dairy chemistry, proteins: basic aspects* (Vol. 1A, 4th
368 edn., pp. 43-85). New York, NY, USA: Springer Science.
369 Pâquet, D. (1989). Revue bibliographique: la fraction proteose peptones du lait. *Le Lait, 69*, 1-21.
370 Pellegrino, L., Battelli, G., Resmini, P., Ferranti, P., Barone, F., & Addeo, F. (1997). Effects of heat
371 load gradient occurring in molding on characterization and ripening of Grana Padano. *Le Lait,*
372 *77*, 217-228.
373 Raats, D., Offek, M., Minz, D., & Halpern, M. (2011). Molecular analysis of bacterial communities in
374 raw cow milk and the impact of refrigeration on its structure and dynamics. *Food*
375 *Microbiology, 28*, 465-471.
376 Resmini, P., Volonterio, G., Prati, F., Pazzaglia, C., & Motti, G. (1982). Caratteristiche del latte e
377 fenomeni rilevati in caldaia nella lavorazione a formaggio Grana-Padano. *Scienza e Tecnica*
378 *Lattiero-Casearia, 33*, 229-264.
379 Rossetti, L., Fornasari, M., Gatti, M., Lazzi, C., Neviani E., & Giraffa G. (2008). Grana Padano cheese
380 whey starter: microbial composition and strain distribution. *International Journal of Food*
381 *Microbiology, 127*, 168-171.
382 Santarelli, M., Gatti, M., Lazzi, C., Bernini V., Zapparoli G.A., & Neviani E. (2008). Whey starter for
383 Grana Padano cheese: effect of technological parameters on viability and composition of the
384 microbial community. *Journal of Dairy Science, 91,* 883-891.
385 Schlimme, E., Clawin-Radecker, I., Einhoff, K., Kiesner, C., Lorenzen, P., Martin, D., Meisel, H.,
386 Molkentin, J., & Precht, D. (1996). Studies on distinguishing features for evaluating heat
387 treatment of milk. *Kieler Milchwirtschaftliche Forschungsberichte, 48*, 5–36.

388 Sheehan, J., Oliveira, J.C., Kelly, A.L., & Mc Sweeney, P.L.H. (2007). Effect of cook temperature on
389 primary proteolysis and predicted residual chymosin activity of a semi-hard cheese
390 manufactured using thermophilic cultures. *International Dairy Journal, 17,* 826-834.
391 Slattery, L., O'Callaghan, J., Fitzgerald, G.F., Beresford, T., & Ross, R.P. (2010). Invited review:
392 *Lactobacillus helveticus* – A thermophilic dairy starter related to gut bacteria. *Journal of*
393 *Dairy Science, 93*, 4435-4454.
394 Stergiadis, S., Seal, C.J., Leifert, C., Eyre, M.D., Larsen, M.K., & Butler, G. (2013). Variation in
395 nutritionally relevant components in retail Jersey and Guernsey whole milk. *Food Chemistry,*
396 *139,* 540-548.
397 Thoma, C., Krause, I., & Kulozik, U. (2006). Precipitation behavior of caseino-macropeptides and
398 their simultaneous determination with whey proteins by RP-HPLC. *International Dairy*
399 *Journal, 16*, 285-293.
400 van Boekel, M.A.J.S., Crijns, C.L. (1994). Behaviour of the proteose-peptone fraction during
401 renneting of milk. *Netherlands Milk & Dairy Journal*, *48*, 117-126.
402 Wedholm, A., Larsen, L.B., Lindmark-Mansson, H., Karlsson, A.H., & Andrén A. (2006). Effect of
403 protein composition on the cheese-making properties of milk from individual dairy cows.
404 *Journal of Dairy Science, 89*, 3296-3305.

Table 1

Content of soluble β-lactoglobulin (β-Lg), α-lactalbumin (α-La), proteose-peptones (PP), small peptides (SP) and free amino acids (FAA) in raw milk samples collected at eight dairies producing Grana Padano cheese in different seasons.

	Spring / Summer n=15				Autumn / Winter n=9			
	mean (mg L^{-1})	min. ± SD[a] (mg L^{-1})	max. ± SD (mg L^{-1})	CV[b]	mean (mg L^{-1})	min. ± SD (mg L^{-1})	max. ± SD (mg L^{-1})	CV
β-Lg	3438	3076 ± 45	3829 ± 37	8	3245	3073 ± 51	3910 ± 30	9
α-La	1152	1105 ± 31	1243 ± 19	3	1121	1056 ± 31	1199 ± 28	4
PP	502	281 ± 25	650 ± 26	24	457	193 ± 30	655 ± 20	29
SP	507	241 ± 23	722 ± 19	36	508	279 ± 21	595 ± 22	19
FAA	76	63 ± 3	90 ± 4	13	75	67 ± 4	89 ± 5	10

[a] SD: standard deviation.

[b] CV: coefficient of variation.

Table 2

Content of free amino acids in 24 samples of raw milk collected at eight dairies producing Grana Padano cheese.

	mean (mg L^{-1})	min. ± SD[a] (mg L^{-1})	max. ± SD[a] (mg L^{-1})	CV[b]
Asp	2.93	2.19 ± 0.13	3.38 ± 0.17	12
Thr	1.43	1.18 ± 0.07	1.78 ± 0.11	11
Ser	1.07	0.83 ± 0.08	1.44 ± 0.09	14
Asn	0.27	n.d[c]	0.53 ± 0.05	58
Glu	42.97	35.3 ± 1.4	53.9 ± 1.6	13
Gln	1.96	0.03 ± 0.00	5.09 ± 0.25	67
Gly	6.75	4.98 ± 0.30	9.43 ± 0.47	16
Ala	3.59	3.02 ± 0.18	4.15 ± 0.21	10
Cit	0.81	n.d.	2.18 ± 0.13	79
Val	2.18	1.02 ± 0.06	3.92 ± 0.20	44
Met	0.09	n.d.	0.23 ± 0.02	89
Ile	0.59	0.33 ± 0.03	0.88 ± 0.09	25
Leu	0.86	0.42 ± 0.04	1.43 ± 0.09	37
Tyr	0.63	0.05 ± 0.00	2.15 ± 0.13	62
Phe	0.55	0.09 ± 0.01	0.90 ± 0.09	43
Gaba	0.04	n.d.	0.25 ± 0.03	167
Orn	0.61	0.47 ± 0.05	0.76 ± 0.08	12
Lys	2.56	1.88 ± 0.11	3.27 ± 0.16	19
His	0.45	0.24 ± 0.02	0.63 ± 0.06	22
Arg	2.92	1.82 ± 0.11	3.64 ± 0.18	17
Pro	2.29	1.86 ± 0.11	3.15 ± 0.16	13

[a] SD: standard deviation.

[b] CV: coefficient of variation.

[c] Not detectable.

437

438 **Captions to figures**

439

440 Fig. 1. Typical HPLC patterns of soluble nitrogen compounds in samples of whole milk (WM), sweet
441 whey (SW) and whey culture (WC). SP, small peptides; PP, proteose-peptones; α-La, α-
442 lactalbumin; β-Lg, β-lactoglobulin; CMP, caseinomacropeptide; BSA, blood serum albumin;
443 Ig, immunoglobulins.

444 Fig. 2. Evolution of the different fractions of soluble nitrogen compounds in whole milk (WM),
445 skimmed milk (SM), sweet whey (SW) and the derived whey culture (WC) samples collected
446 from 24 different Grana Padano cheese-makings. Error bars represent total ranges of values,
447 columns indicate mean values, figures are coefficient of variation values. SP, small peptides;
448 PP, proteose-peptones; α-La, α-lactalbumin; β-Lg, β-lactoglobulin; CMPs,
449 caseinomacropeptides; FAA, free amino acids.

450 Fig. 3. Box whisker plots of the different fractions of soluble nitrogen in 40 samples of natural
451 whey culture collected at six Grana Padano cheese dairies in different days. β-Lg, β-
452 lactoglobulin; α-La, α-lactalbumin; PP, proteose-peptones; SP, small peptides; CMPs,
453 caseinomacropeptides; FAA, free amino acids.

454 Fig. 4. Sweet whey fermentation to prepare the natural whey culture at a Grana Padano cheese
455 dairy. A: standard plate count (SPC), pH, and temperature gradient; B: behavior of the
456 different soluble nitrogen compounds. β-Lg, β-lactoglobulin; α-La, α-lactalbumin; PP,
457 proteose-peptones; SP, small peptides; CMPs, caseinomacropeptides; FAA, free amino acids.

458 Fig. 5. Sweet whey fermentation to prepare the natural whey culture at a GP cheese dairy:
459 behavior of individual free amino acids. A: threonine, serine, glycine, leucine; B: methionine,

460 tyrosine, phenylalanine; C: glutamic acid, glutamine, γ-amino butyric acid; D: arginine,
461 ornithine, citrulline.